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1. TNA Provided

Name of the TNA project	Name of TNA user	Organisation of TNA user	Country of TNA user	Installation from the RI	Start date	End date	Number of units of access provided
Experimental infection of Red fox (Vulpes vulpes) with a Mycobacterium bovis strain naturally virulent	Ana Maria Balseiro	University of León	Spain	Nancy Rage	September 2018	December 2019	1
Assessing the ferret as a bovine tuberculosis transmission model (FerreTub)	Fred Quinn	University of Georgia	USA	Nancy Rage	September 2020	July 2021	6

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2. Final reports of each TNA provided

2.1 TNA 1

Experimental infection of Red fox (Vulpes vulpes) with a Mycobacterium bovis strain naturally virulent

Summary

Mycobacterium bovis excretion in urine and faeces in naturally infected foxes has been described but no experimental data are available on the pathogenesis of *M. bovis* infection in foxes and the associated risk of excretion. To validate an experimental infection protocol by the oral route, 12 captive foxes (6 females and 6 males) were infected with an *M. bovis* field isolate ($1.5x10^7$ CFU). Immunology, bacteriology and pathology protocols aligned with those already used with *M. bovis* experimentally infected European badgers and ferrets were performed 12 weeks post-infection. At post-mortem, only few macroscopic lesions were observed. Histology showed small granulomas within the lymph nodes, tonsils, liver and lung from a small number of foxes, with the presence of scarce acid-fast bacilli. All 12 foxes had at least four PCR positive samples (out of the 23 tested), and 11 foxes had at least one culture positive sample. The culture negative fox was PCR positive in both retropharyngeal and the mesenteric lymph nodes, in line with the results of the other animals. *M. bovis* was detected by PCR in the bladder of 3 foxes at 82 days post-infection and, during the experiment, in faeces of 9 foxes and in the oropharyngeal mucus of 3 individuals. This experimental oral infection was therefore able to reproduce the pattern of infection observed in naturally TB-infected foxes in the wild and to demonstrate the risk of mycobacteria excretion by TB infected foxes.

Introduction & Aim of the project

Animal Tuberculosis (TB) caused by *Mycobacterium bovis* is one of the most important animal diseases in Europe. Its control, if not eradication, is urgent for the farming industry and for the public health (zoonotic infection) but also for the environment because some wildlife populations are infected. In spite of the application of control programmes since several decades, the prevalence of TB in the UK and Ireland are still the highest in Europe. This disease also emerges in local areas in France, Spain and Portugal. Wildlife (badger and wild boar) can play a role of reservoir for TB, as demonstrated in the UK, Ireland and Spain. To assess the risk of transmission spill over of the disease from wildlife to livestock, the role of wildlife species has to be better understood.

The red fox (*Vulpes vulpes*) is usually considered as a minor spill over host of TB in Europe. In the early 2000s, only 3% tested of foxes were infected by *M. bovis* in a Great Britain area where cattle TB prevalence and infection in badgers was high, and only one animal presented visible lesions. But, more recently, higher TB prevalence was observed in red foxes in Iberian Peninsula (up to 26.9%). In these areas, TB circulates in a multi-host species system and this raises questions about the putative role of the fox in local TB epidemiology and the risks of transmission from foxes to cattle. *M. bovis* excretion in fox faeces, urine and tracheal mucus was originally estimated as very low. However, *M. bovis* was detected in the clinical samples of four infected red foxes captured in a highly prevalent area in France, where cattle, badgers, wild boars and even roe deer and red deer were also affected by the disease. Thus, the aim of this proposal was to develop of a reproducible infection model in foxes that will provide data

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on the pathogenicity and excretion data in this species, suitable for modelling risk transmissions in Spain (hence this project proposal by SERIDA/University of León) and France, and for testing experimentally the protective efficacy of vaccines in captive animals. This approach constitutes the first experimental infection model with *M. bovis* in red foxes.

Materials & Methods

Twelve foxes of either sex and 1-4 years of age were infected with a French field strain of *M. bovis* by the oral route (1 mL of $1.5.10^7$ CFU/mL solution administered orally in a piece of raw beef). The rational for this route was based on field data suggesting the oral route as potential infection due to the high presence of intestinal tuberculosis.

Blood samples were taken at D0 and then every 4 weeks to monitor the immune response of infected foxes by different methods (IFN-γ release assay on whole blood and PBMC, and serological test using MPB83 antigens)

Twelve weeks after infection, the animals were anesthetized, euthanized and submitted to a detailed post-mortem examination with registration of any macroscopic lesion and collection of 23 tissue samples for histopathological analysis. The presence of *M. bovis* was also assessed in these tissues by PCR and culture.

Bacterial excretion monitoring was performed by PCR and culture on oropharyngeal swabs, urine and faeces collected every 4 weeks during the experiment.

Ethical license

The experimental procedure has been approved by the local ethical committee for animal experimentation n°16 and the respective license granted by the French "Ministère de l'Enseignement Supérieur et de la Recherche" on 10/02/2019 (APAFIS#16237-2018072316235926v4).

Results

The back titration of the challenge strain gave similar result to the expected dose, validating the challenge. No clinical signs were observed during the 12 weeks of experiment.

All fox had positive results to IFN-γ release assay on whole blood during the experiment and 10 at the last time-point, 12 weeks post-infection. Ten foxes had positive serological results during the experiment, with 9 foxes positive at the last time-point.

At post-mortem, only few macroscopic lesions were observed. Histology showed small granulomas within the lymph nodes, tonsils, liver and lung from a small number of foxes, with the presence of scarce acid-fast bacilli.

All 12 foxes had at least four PCR positive samples (out of the 23 tested). The infection was confirmed by culture in 11 foxes. The culture negative fox was PCR positive in both retropharyngeal and the mesenteric lymph nodes, in line with the results of the other animals, where the retropharyngeal lymph nodes, tonsils and mesenteric lymph nodes were the most frequently infected tissues (infection found in 11/12, 10/12 and 6/12, respectively).

M. bovis was detected by PCR in the bladder of 3 foxes 12 weeks post-infection and, during the experiment, in faeces of 9 foxes (two confirmed by culture) and in the oropharyngeal mucus of 3 individuals.

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Conclusions

The 12 foxes were all diagnosed positive for *M. bovis* in at least one tissue and thus we can conclude that they were successfully orally challenged with a field *M. bovis* strain. No clinical impact and very few macroscopic and histological lesions were observed. In a few animals, *M. bovis* was detected in faeces and in the oropharyngeal mucus during the 12 weeks PI and in the bladder at post-mortem. This experimental oral infection was able to reproduce the pattern of infection observed in naturally TB-infected foxes in the wild and to demonstrate the risk of mycobacteria excretion by TB infected foxes revealing that they may contribute to the maintenance of TB in its multi-host epidemiological system.

2.2 TNA 2

Assessing the ferret as a bovine tuberculosis transmission model (FerreTub)

Summary

To eradicate bovine tuberculosis (TB) caused by *Mycobacterium bovis*, major wildlife reservoirs of infection such as badgers (*Meles meles*) must be controlled. Oral vaccines (BCG) for badgers are still in development (Lesellier et al., 2020) and protection measured experimentally as a reduction of the TB lesions severity induced by a high dose of *M. bovis* in vaccinated animals is not associated with the decrease of blood markers of infection seen in the field (Carter et al., 2012; Gormley et al., 2017). Physiological transmission conditions are difficult to reproduce in badgers as would require months at high-level containment, but are possible in other laboratory-adapted mustelids, ferrets (*Mustela furo*), as shown for *M. tuberculosis* (Gupta et al., 2022), and after oral *M. bovis* inoculation (Qureshi et al., 2000). This project aimed at studying blood biomarkers of *M. bovis* transmission in ferrets. Sixteen ferrets, were experimentally infected with *M. bovis* by the intra-tracheal route and developed TB typical granuloma and strong immune responses from 6 weeks post-infection until the end of the study, nine months later. The 24 in-contact animals housed with them developed no disease but consistent milder serological and cellular blood immune responses showing exposure to *M. bovis*. The project demonstrated for the first that blood biomarkers reflect *M. bovis* transmission by contact housing of experimentally infected ferrets with naïve ferrets. On this basis, the protective effect of TB oral vaccines could be evaluated in the future in ferrets as a reduced transmission measured by blood biomarkers, as in field badgers.

Introduction & Aim of the project

Bovine Tuberculosis (bTB) caused by *Mycobacterium bovis* (*M. bovis*) remains one of the most important animal diseases worldwide, and it contributes to a significant lethal burden of tuberculosis in humans. In Europe, the prevalence of TB can be very high in livestock (more than 10% of herds in the UK and around 5% in Ireland, whereas Officially TB free countries demonstrate a prevalence below 0.1% persistently) and is rising in local areas of France, Spain and Portugal, with significant financial, social and environmental impacts. TB eradication in cattle is therefore urgent with control of infection from all main sources, including wildlife reservoirs for TB. Developing oral veterinary vaccines for badgers (*Meles meles*) against TB (Lesellier et al., 2020; Gormley et al., 2021) is therefore an international endeavour to reduce in a sustainable manner the long-term risks of transmission to cattle.

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Current TB badger models generally utilize high doses of laboratory-grown *M. bovis* bacilli delivered in single administration infection methods such as intra-tracheal or intra bronchial instillation of bacteria suspended in liquid. Such methodology accelerates the progression of the disease so that it becomes compatible with laboratory use in wild animals at high containment level (within 12 weeks instead of months). In these conditions, readouts of the studies are qualitative/quantitative assessments of pathology/bacteriology at necropsy, as severe signs of advanced disease that can be quantified and should be reduced to control the epidemic. However such an advanced stage of disease remains rare in badgers (Corner et al., 2009); hence, measuring this end-of-the-spectrum outcome does not match the epidemiological objective of reducing infection rate. A high challenge dose may also overwhelm the protective effect granted by vaccines compared with normal infection conditions.

In the field, the vaccination protective effect can be measured in live cohorts of vaccinated badgers, mainly through the decrease of blood markers of infection (Carter et al., 2012; Gormley et al., 2017) in a more efficient way than by trapping/conducting post-mortem on a trapped subset of vaccinated/control badgers after several years of deployment. Removing the vaccinated badgers for post-mortem also stops the long-term protective benefit in infected badger populations.

The high dose of *M. bovis* used for challenging vaccinated and naïve badgers in experimental studies is not associated with the decrease of blood markers of infection seen in the field. A transmission study in experimental conditions, with detectable immune responses more similar to those seen in the field, would therefore benefit the evaluation of vaccine efficacy studies. Physiological transmission conditions are difficult to reproduce in badgers as would require months of *M. bovis* exposure at high-level containment, but are possible in other laboratory-adapted mustelids, ferrets (*Mustela furo*), as shown for *M. tuberculosis* (Gupta et al., 2022), and after oral *M. bovis* inoculation (Qureshi et al., 2000).

This project aimed at studying blood biomarkers of *M. bovis* transmission in ferrets during 9 months exposure between experimentally infected ferrets and in-contact naïve ferrets.

Materials & Methods

Animals

In this project, 40 male ferrets (castrated and vaccinated against rabies) were transferred to the ANSES-Nancy facility from Denmark (Euroferrets). The animals were housed at all time at level 3 containment in two separate rooms, including for the first two weeks of acclimation. The animals were divided in four groups of 10 animals each (two groups per room). Animals were free to range, feed and drink at libitum, climb, hide, in large and interconnected house-made cages following the European 2010/63 Directive.

In the course of the study, four animals died (two by accident and two with unexplained severe TB), and were replaced by four new ferrets on the 13/08/2021. Two of these animals were IT animals, and the new animals were in-contacts for only 16 weeks.

Inoculation

At D0, sixteen ferrets (4 groups of 4 animals - 4 animals per group of 10) were experimentally infected with approximately 10^3 Colony Forming Units (CFU) of *M. bovis* (AF2122/97) in 500µl suspension. Under general anesthesia, each ferret was inoculated by the intra-tracheal route (IT group), using single-use catheters positioned in the trachea with a laryngoscope. The

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twenty-four naïve ferrets (4 groups of 6 animals- 6 animals per group of 10) were then housed with their in-contact cage group for 9 months.

On 9 time-points, approximately every 4-6 weeks (disruptions in time-tables occurred because of COVID-9), all the ferrets were anesthetised by isoflurane delivery to the nose, for collection of blood and of tracheal mucus. Blood was obtained from the anterior vena cava with a 21g needle, in heparinised vacutainers in order to separate plasma and PBMCs. Plasma and tracheal mucus were stored frozen before analysis. PBMCs were analysed fresh.

At the end of the study, the ferrets were deeply euthanised and submitted to extensive post-mortem. Tissues were collected to quantify the severity of lesions (by histology) scored as in previous studies for TB infected badgers (Lesellier et al., 2011) and ferrets

MPB83 specific serological responses were measured in plasma by ELISA with a mustelid modified Idexx TB test.

IFN-γ responses were measured by ELISPOT (Mabtech) using fresh PBMCs stimulated with PPD-B (30µg/ml- Lelystadt), PPD-A (30µg/ml- Lelystadt), ESAT-6 (5µg/ml- Lionex), CFP-10 (5µg/ml- Lionex), ConA (5µg/ml- Sigma), or growing medium without antigen (RPMI completed by foetal calf serum, NEAA and β-mercapto-ethanol) for 16h at 37degrees and 5% CO2 atmosphere as described in (Lesellier et al., 2006).

Tracheal mucus swabs and brocho-alveolar lavages (BAL) were tested by RT-PCR to quantify the presence of *M. bovis* as described in Lesellier et al., 2019. The analysis of tissues by RT-PCR is still on-going.

In order to quantify the TB lesions size and localisation in the lungs, MRI images of the formalin fixed lungs were acquired as reported in Deliverable D11.1 (TNA 5, INRAE-PIXANIM).

Ethical license

Number of the license granted by the French Ministry of Academia, Research and Innovation: APAFIS#8234-201610211414126 v5.

Results

IT infected ferrets

TB was confirmed in most of the IT ferrets (14/16 ferrets). As observed in previous studies with IT infection of badgers and ferrets with *M. bovis*, only mild TB visible lesions were observed at the surface of the lungs and in lymph nodes. Most advanced stages of lesions with mineralisation were not observed in any animal and tissues.

TB lesions were visible by MRI scanning in formalin fixed tissues of some animals. An example is shown below (Figure 1) of ferret #15's lesions, which also displayed the most severe histological lesions (Table 1). Further analysis of the images is on-going by PIXANIM to quantify the volume of the lesions in all the animals.

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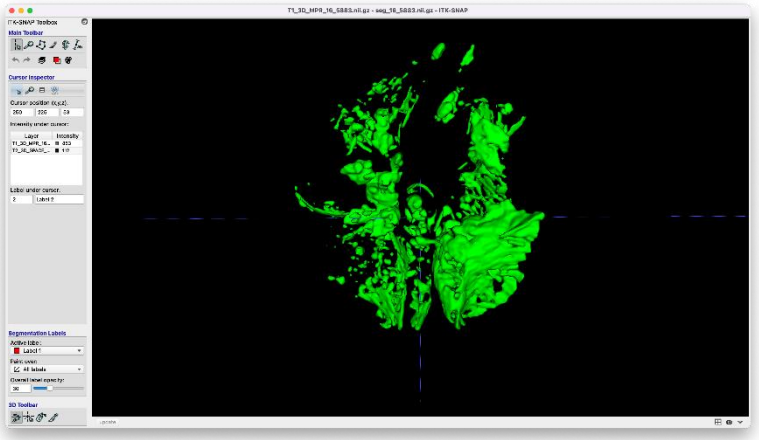

Figure 1: MRI scan of formalin fixed ferret lung with TB lesions using a Siemens 3 Teslas (T) Magnetom Verio scanner.

M. bovis caused mild typical TB granulomatous lesions, scored as previously for experimentally infected badgers and foxes. As in the previous studies, no multinucleated cells were observed.

Lesions were observed mostly in the lungs, but also in the mesenteric lymph nodes (Table 1).

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		Histology scores			PCR	
N° F	Infection route	Lung tissue	LN		BAL	Mucus trachéal
1	IT					
2	IT	1				
3	iN-C 40w					
4	iN-C 40w					
5	iN-C 40w					
6	iN-C 40w					
7	IT	2				
8	IT	1				
9	iN-C 40w					
10	iN-C 40w	1				
11	iN-C 40w					
12	iN-C 40w					
13	iN-C 40w					
14 (4232)	IT for 11w					Pos (T2)
15 (5883)	IT for 11w	4	3	Mesenteric		
16 (6967)	IT for 10w	2	2	Retro		
17	iN-C 40w					
18	iN-C 40w					
19	iN-C 40w					
43	iN-C last 16w					
44	iN-C last 16w					
20	IT	2	1	Mesenteric		
21	iN-C 40w					
22	iN-C 40w					
23	IT	2	3	Mesenteric		
24	iN-C 38w					
25	IT	2	2	Mesenteric	Pos	
26	IT for 6 w	Mort accidentelle à 6 semaines/ Pas autopsié				
27	iN-C 40w					
28	iN-C 40w					
29	iN-C 40w					Pos (T1)
30	IT	2	2	Ant media		
41	iN-C last 16w	3	3	Mesenteric		
31	IT		2	Mesenteric		
32	iN-C 40w					
33	iN-C 40w					
34	IT	2	2	Retropha	Pos	Pos (T4)
35	iN-C 40w					Pos (T5)
36	iN-C 40w					
37 (5325)	IT for 13w	2	2	Mesenteric		Pos (T3)
38	IT		4	Mesenteric et mediast		Pos (T1-T6)
39	iN-C 40w					
40	iN-C 40w					
42	iN-C last 16w	1				

Table 1: Scoring of histological lesions in tissues obtained at PM and RT-PCR analysis of tracheal mucus and BAL in ferrets infected experimentally by the intracheal route (IT) and b transmission (in-C).

M. bovis DNA was identified in the broncho-alveolar lavages (BAL) collected at PM or in the tracheal mucus collected at each time-point during the course of the study, mostly in the IT ferrets.

Immune responses were measured at regular intervals throughout the study (Figure 1). The IT group produced strong cellular (IFN-γ) responses that peaked at 6 weeks post-challenge and decreased afterwards. The kinetic of the responses mirrored those seen in *M. bovis* infected badgers. A major difference in the ferret responses compared with those of badgers was the ESAT-6 response almost as high as the PPD-B response, whereas the badger ESAT-6 responses generally remained low throughout the experimental TB infection, independent of the *M. bovis* strain or the badger group.

In-contact ferrets

Most of the animals did not present TB typical lesions at post-mortem (Table 1). Bacteriological analysis of PM tissues is still pending.

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In-contact animals generally presented no detectable granuloma in tissues but consistently increased immune responses (Figure 2).

As in the IT group, the animals' IFN-γ responses peaked at - weeks post-challenge, but at a moderate level.

The serological responses as determined by recognition of MPB83-specific IgG in plasma were increased in a majority of these animals from 6 weeks post-challenge. In particular, MPB83 specific serological responses developed as rapidly as in the experimentally infected animals although at lower levels. Antigen specific cellular immune responses also were also measured in the in-contact group, IFN-γ and IL-2 based against *M. bovis* tuberculin, but also DIVA antigens ESAT-6 and CFP-10.

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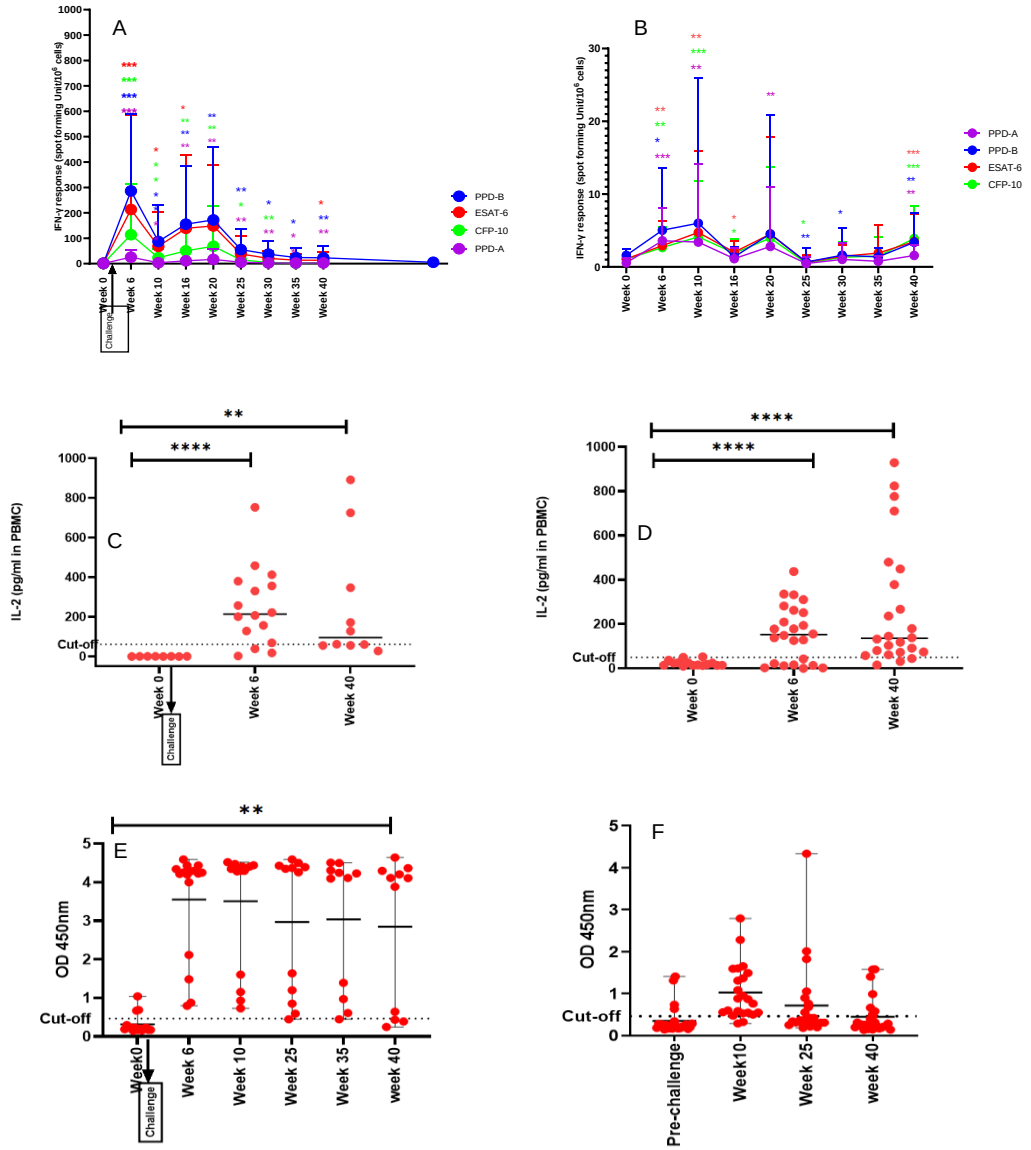

Figure 2: Cellular responses to PPD-B, ESAT-6, CFP-10 and PPD-A (IFN-γ response of IT group (A) and in-contact group (B), IL-2 response of IT group (C) and in-contact group (D)) and detection of MPB83 specific IgG responses in IT group (E) and in-contact group (F).

Conclusions

This project aimed at measuring blood biomarkers for a naturel TB infection model based on animal-to-animal transmission for 9 months in ferrets. Ferrets (*Mustela furo*) were used as laboratory surrogates for badgers (*Meles meles*), given their genetic homologies (carnivore family mustelids). Ferrets are natural hosts for TB in the wild (mostly in New Zealand) and are used for studying pulmonary diseases in laboratory conditions. Such data are required for improving the protocols of experimental studies and for evaluating protective effects of vaccines under relevant challenging conditions.

M. bovis experimentally infected ferrets by the IT route developed a detectable disease in most of the animals at post-mortem 39 weeks post-challenge, with mild histology scores and symptoms, as seen previously in experimentally infected ferrets (Qureshi et al., 2000) and badgers (Lesellier et al., 2020). In the rare animals with the most severe lesions, a detection

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of the lesions by MRI was possible, as in TB infected badgers. Cellular and serological responses also developed, divided in two distinctive high and low responder groups. Detectable *M. bovis* excretion was rare in tracheal mucus collected approximately every 4 weeks throughout the study. Detecting M. bovis in clinical samples remains challenging and is not considered sufficient to quantify the excretion of infectious *M. bovis*.

In-contact animals generally presented no detectable granuloma in tissues but consistent low levels immune responses. In particular, the serological responses (MPB83 specific IgG responses in plasma) were elevated in most animals by six weeks post-challenge, and remained high through the course of the study. Antigen specific cellular immune responses also increased (IFN-γ and IL-2 based against *M. bovis* tuberculin, but also DIVA antigens ESAT-6 and CFP-10).

Such blood markers can also be measured in field badgers and were found significantly decreased in vaccinated live cohort of badgers compared with non-vaccinated ones, both after intramuscular BCG delivery (Chambers et al., 2011; Carter et al., 2012) and oral delivery (Gormley et al., 2017; Gormley et al., 2021).

TB experimental transmission studies are rarely conducted, given the long duration of the studies and the large number of animals required. They however provide unique opportunities for exploring the peripheral responses of animals more “naturally” exposed to low repeated doses of *M. bovis*, and therefore for developing sensitive tests at early stages of exposures to *M. bovis*. This study demonstrated for the first time that transmission by contact housing of experimentally infected ferrets with naïve ferrets can stimulate blood biomarkers, in particular serological responses. Such responses measured in blood are gaining a growing interest, as potentially more sensitive at early stages than previously anticipated and sustainable for large scale testing for surveillance and for testing vaccine efficacy. Further testing of the samples generated during this study will be conducted in the near future with newly identified antigens to enhance sensitivity further.

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